Therapeutic targeting of protein S-acylation for the treatment of disease

Niall J. Fraser[1*], Jacqueline Howie[2], Krzysztof J. Wypijewski[2], and William Fuller[2*]

1. School of Medicine, University of Dundee, UK.
2. Institute of Cardiovascular and Medical Sciences, University of Glasgow, UK.

* To whom correspondence should be addressed:

NF: Mailbox 12, Level 5, Lab Block, Ninewells Hospital and Medical School, University of Dundee, Dundee, DD1 9SY. Phone: 01382 383950, n.fraser@dundee.ac.uk, ORCID: 0000-0001-7349-5647.
WF: Room 214, Sir James Black Building, University Avenue, Glasgow G12 8QQ, UK. Phone: 0141 330 3228, will.fuller@glasgow.ac.uk, @FullerLabGlas, ORCID: 0000-0002-5883-4433.

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Abstract

The post-translational modification protein S-acylation (commonly known as palmitoylation) plays a critical role in regulating a wide-range of biological processes including cell growth, cardiac contractility, synaptic plasticity, endocytosis, vesicle trafficking, membrane transport and biased-receptor signalling. As a consequence, zDHHC-protein acyl transferases (zDHHC-PATs), enzymes that catalyse the addition of fatty acid groups to specific cysteine residues on target proteins, and acyl proteins thioesterases (APTs), proteins that hydrolyse thioester linkages, are important pharmaceutical targets. At present, no therapeutic drugs have been developed that act by changing the palmitoylation status of specific target proteins. Here, we consider the role that palmitoylation plays in the development of diseases such as cancer and detail possible strategies for selectively manipulating the palmitoylation status of specific target proteins, a necessary first step towards developing clinically-useful molecules for the treatment of disease.

Introduction

Protein S-acylation, the reversible covalent attachment of fatty acids (chain length ranging between 16 and 20 Carbons (C)), typically palmitic acid (C = 16), to the thiol groups of specific cysteine residues in substrate proteins, has recently emerged as an important and common post-translational modification in a variety of tissues regulating a diverse range of physiological processes including cell growth, cardiac contractility, synaptic plasticity, endocytosis, trafficking and biased signalling [1-4]. Protein S-acylation occurs dynamically and reversibly in a manner analogous to protein phosphorylation [5], is catalysed by a family of zinc-finger and DHHC-motif containing protein acyltransferases (zDHHC-PATs) [1,3] and reversed by acyl protein thioesterases (APTs) [6]. Many different classes of protein have been identified as targets for acylation, including G-proteins [7], ion channels [8-13], transporters [14-16], pumps [17, 18], receptors [19-23] and protein kinases [24-26]. Acylation induces changes in the local structure (e.g. [27]) and, therefore, function of the intracellular regions of target proteins through their recruitment to the surface of a membrane bilayer via the acylated cysteine, and results in either a change in the activity and/or localisation of the modified protein [1,3].

zDHHC-PATs are zinc-finger-containing enzymes characterised by a cysteine-rich region with a conserved Asp-His-His-Cys (DHHC) motif [1,3] within the active site [28]; there are 23 human isoforms. zDHHC-PATs are expressed throughout the secretory pathway (including the cell surface) [29]. They typically have 4 transmembrane (TM) domains, with a conserved ~50 amino acid cytosolic core between TM2 and 3 which contains the DHHC motif. In contrast, the intracellular amino and carboxyl termini are poorly conserved, and likely contribute to zDHHC-PAT isoform substrate selectivity [3, 30].

In contrast, APTs are soluble proteins that belong to the α/β-hydrolase family of serine hydrolases, which all have a characteristic active site serine essential for enzyme activity [6, 31-32]. The first acyl protein thioesterase to be discovered was APT1 [33], which has since been shown to depalmitoylate a diverse range of proteins including Gαs, endothelial nitric oxide synthase (eNOS) and SNAP-23 but not caveolin [34-36]. Subsequent bioinformatics analysis revealed a related depalmitoylase with 64% sequence identity to APT1 called APT2

[37]. For many years, it has been thought that APT1 and APT2 were the only enzymes that could remove palmitate from cytosolic cysteine residues but recently it has been shown that the ABHD17 family of serine hydrolyses can depalmitate N-Ras [38]. In humans, the serine hydrolase superfamily has more than 100 members [31], many of which have as yet unknown substrate specificity suggesting that there may be other depalmitoylases waiting to be discovered.

Protein S-acylation and disease

Given the vast number of cellular events regulated by palmitoylation, it is not surprising to discover that misregulation of palmitoylation is a significant causal factor in a range of human pathologies. The link between protein acylation and disease is probably best established for cancer [39-40], for which there are several established examples. For instance, non-small cell lung cancer growth is zDHHC5-dependent [41], and palmitoylation of EZH2 by zDHHC5 is required for malignant development and progression of p53-mutant glioma [42]. zDHHC3 has been shown to play a vital role in regulating breast tumour growth, oxidative stress and senescence [43]. Development of EGFR mutated non-small cell lung cancer is dependent upon EGFR palmitoylation [44]. A mouse that developed a spontaneous truncation mutation in its *Zdhhc13* gene had increased susceptibility to skin carcinogenesis compared to wild-type animals suggesting that zDHHC13 has a protective role against the development of skin cancer [45]. Palmitoylation of the nuclear transcription regulator SCP1 causes it to be located at the plasma membrane instead of the cell nucleus, which impedes its ability to promote both angiogenesis and tumour growth [46]. Palmitoylation is also known to regulate a wide range of neurological processes, and plays a causal role in a range of neurodegenerative diseases [47] including Alzheimer's disease [48]. Last, blocking palmitoylation of the STING (stimulator of interferon genes) protein at a transmembrane cysteine reduces STING-mediated inflammatory cytokine production in both human and mouse cells, suggesting a possible therapeutic option for treating autoinflammatory disease [49]. These examples indicate the potential scope of pharmacological manipulation of protein palmitoylation for the treatment of disease.

A full consideration of all diseases caused by aberrant protein acylation is beyond the scope of this review. Instead, three case studies will be presented here in depth explaining the regulatory role played by palmitoylation in several cellular processes, and how diseases related to these physiological events may potentially be tackled through modulating the acylation status of particular target proteins.

Case study 1: Oncogenic Ras

Ras proteins function as molecular switches transmitting signals from cell surface growth factor receptors to intracellular effector proteins cycling between inactive (GDP bound) and active (GTP) states [50, 51]. On binding GTP, Ras undergoes a conformational change that enables it to interact with and regulate proteins such as Raf (signalling cascade RAF-MEK-ERK-MAPK) [52, 53] and the P110 sub-unit of PI3K (PI3K-AKT-mTOR) [54, 55]. Ras GDP-GTP exchange is promoted by guanine nucleotide exchange factors (GEFs, e.g. SOS1) whereas GTPase activating proteins (GAPs) stimulate GTP hydrolysis, promoting Ras inactivation [56]. In humans, the genes HRAS, NRAS and KRAS encode three closely related proteins that are frequently mutated in cancer, the most common of which is K-RAS (86% of all RAS mutant cancers) with smaller contributions from N-RAS (11%) and H-RAS (3%), that play a pivotal role in driving unregulated cell growth [51, 57-58]. The K-RAS gene gives rise to two splice variants (K-RAS4A, 4B), both of which have their own distinct biochemical properties. Ras oncogenic activity requires the protein to be localised to the inner face of the plasma membrane suggesting strategies that cause Ras to be mistargeted within the cell may be effective in treating cancer [59, 60].

Ras membrane association is facilitated by two lipid post-translational modifications – farnesylation (all Ras isoforms) and palmitoylation (H-Ras, N-Ras and K-Ras4A only) – at specific cysteine residues located at the protein's C-terminus [61, 62]. As all Ras proteins are modified by farnesylation [61, 62], and this post-translational modification is required for correct membrane localisation of Ras [63], considerable effort has been expended trying to identify inhibitors of farnesyl transferase (FTase, the enzyme responsible for Ras farnesylation), two of which (Lonafarnib, Tipifarnib) made it to phase III clinical trials. Although both drugs

showed encouraging preclinical activity [64, 65], they were found to be ineffective in patients who had pancreatic, colorectal or lung cancer caused by oncogenic K-Ras [66, 67]. This lack of clinical efficacy was due to the compensatory ability of a related enzyme geranylgeranyl transferase I to prenylate K-Ras and N-Ras in the presence of a FTase inhibitor [68]. As H-Ras is prenylated by FTase alone, however, it has been possible to use Tipifarnib to treat those squamous cell head and neck cancers caused by oncogenic H-Ras, thus demonstrating that the strategy of mistargeting Ras is clinically effective [69]. As yet, the therapeutic potential of blocking Ras palmitoylation has yet to be determined.

Ras is palmitoylated in the Golgi by an enzymatic complex consisting of zDHHC9 and GCP16 [70], which were identified as the human orthologs of Erf2 and Erf4 respectively, proteins previously shown to be responsible for Ras palmitoylation in yeast [71, 72]. Although the palmitoylation reaction is carried out by zDHHC9, the stability and catalytic activity of the enzyme is dependent on the presence of GCP16 [70]. Ras palmitoylation occurs after it has been farnesylated, and this second lipid modification increases the affinity of Ras for membranes by more than 100-fold [73, 74]. Once palmitoylated, Ras is trafficked from the Golgi to the plasma membrane [5]. Palmitoylation of N-Ras is essential for both EGF mitogenic signalling in mouse embryonic fibroblasts [75] and the development of leukaemia [76]. Furthermore, downregulation of zDHHC9 expression in somatostatin-positive interneurons has been shown to cause reduced membrane localisation of Ras [77]. All together, the available evidence suggests that those Ras isoforms that get palmitoylated can be prevented from associating with the plasma membrane through inhibiting zDHHC9 activity. This said, however, developing an active site inhibitor of zDHHC9 as a means of blocking Ras palmitoylation is unlikely to be useful in the clinic as such a molecule would be expected to cause significant serious neurological side effects. Mutations in the gene encoding zDHHC9 are known to cause mild-to-moderate intellectual disability, seizures as well as speech and language impairment [78-81]. Furthermore, a *Zdhhc9* knockout mouse has recently been shown to have seizure-like activity with increased frequency and amplitude of both spontaneous and inhibitory post-synaptic currents [82]. If a molecule that prevents Ras from interacting with the

zDHHC9-GCP16 complex can be identified, however, it may be possible to selectively block Ras palmitoylation.

When the crystal structure of H-Ras was first reported, with the exception of the nucleotide-binding site, no large hydrophobic pockets on the surface of the protein that could readily accommodate small molecules were observed [83], suggesting that efforts to drug Ras itself would prove challenging, as indeed has been the case. In recent years, however, several groups have identified both small molecules [84-88] and peptides [89-90] that bind within shallow pockets on the surface of Ras preventing it from interacting with either SOS1 [84-85, 88-90] or c-Raf [86, 88], reducing both downstream signalling and ultimately cell proliferation. Successful targeting of the contact interface between Ras and both SOS1 and C-Raf suggests that it may also be possible to drug other protein-protein interactions made by Ras. For example, palmitoylation of Ras by zDHHC9-GCP16 must entail a physical contact between the enzyme complex and its substrate. The region of Ras surrounding its palmitoylation site is recognised by and likely binds within the enzyme's active site [91, 92]. It is also conceivable, however, that Ras contacts the zDHHC9-GCP16 enzyme complex at (a) region(s) distinct from its palmitoylation site. If indeed this is the case, once those regions of Ras that interact with either zDHHC9 and/or GCP16 have been identified, it should be possible to develop molecules and/or peptides that block this interaction preventing H-, N- and K-Ras4A from being palmitoylated.

Selective blockade of Ras palmitoylation may prove to be a better therapeutic strategy than blocking the interaction between Ras and individual effector enzymes as Ras mislocalisation will block all downstream signalling events. This said, the strategy of targeting Ras palmitoylation will have no effect in those cancers caused by oncogenic KRAS4B as this splice variant associates with the plasma membrane via electrostatic interactions [93]. Furthermore, recent evidence has shown that in the absence of palmitoylation, K-Ras4A is still able to associate with the plasma membrane via two clusters of positively charged residues located within its hypervariable region [94], which suggests that targeting Ras palmitoylation for the treatment of cancer may only be a viable strategy for H- and N-Ras isoforms.

Case study 2: MC1R and melanomagenesis

The melanocortin-1 receptor (MC1R), a G-protein coupled receptor, is the key regulator of hair and skin pigmentation in people [95, 96]. Following exposure to ultraviolet radiation, the peptide α-melanocyte-stimulating hormone (α-MSH) binds to and activates MC1Rs in melanocytes (specialised skin cells), which results in cAMP signalling, melanin production and enhanced DNA repair [95-98]. Several non-synonymous single nucleotide polymorphisms (SNPs) in the MC1R gene have been discovered, and a group of them termed red hair colour (RHC) variants are associated with red hair colour, fair skin and poor tanning ability as well as increased risk of developing melanoma [99-103]. The MC1R has recently shown to be palmitoylated at C315 by zDHHC13 following exposure to UV-light [104]. Interaction between zDHHC13 and MC1R was promoted by phosphorylation of zDHHC13 at S8 by ATR (a kinase which is a central effector of the UVB response) [105]. MC1R palmitoylation was essential for signalling through the receptor, and was required for increased pigment production, ultraviolet-B-induced G1-like cell cycle arrest as well as control of senescence and melanomagenesis both *in vitro* and in *vivo* [104]. All together, these results show that MC1R palmitoylation is required for the receptor's tumour suppression activity. In a follow-up study, the same group of researchers sought to address the question as to whether or not therapeutic interventions that enhanced MC1R signalling could reverse the increased melanoma risk associated with the RHC receptor variants [106]?
It was found that targeted expression of zDHHC13 in melanocytes in C57BL/6J-MC1R^{RHC} mice inhibited melanomagenesis. Furthermore, administration of the selective APT2 thioesterase inhibitor ML349 [107-109] increased MC1R signalling and repressed UVB-induced melanamogenesis both *in vitro* and *in vivo*. Although these experiments have generated highly interesting proof of concept data neither therapeutic strategy (targeted cell-specific zDHHC13 overexpression, specific inhibition of a thioesterase that has numerous substrates) will be clinically useful. If a molecule that selectively blocks the association of APT2 and MC1R could be identified, however, it may be useful in helping to protect redheads against harmful, UV-containing sunlight.

Case study 3: Checkpoint blockade therapy

Immune checkpoints are inhibitory signalling pathways that regulate the magnitude and duration of T cell immune responses in peripheral tissues and, as a consequence, are essential for maintaining self-tolerance [110]. One such checkpoint is the interaction that occurs between programmed-death ligand 1 (PD-L1) and its receptor programmed cell death 1 (PD-1), which contributes to the delicate regulatory balance between T-cell activation and tolerance. PD-1 is mainly found on the surface of activated T cells whereas PD-L1 is normally expressed on the surface of antigen presenting cells but can also occur on the surface of cancerous cells where it helps them avoid detection by the immune system. The vast majority of tumour infiltrating lymphocytes (TILs) isolated from prostrate and melanoma biopsies have been shown to have high PD-1 expression levels [111, 112]. Furthermore, PD-L1 is commonly upregulated in a wide-range of cancers including melanoma, ovarian, haematological and non-small-cell lung cancer [113]. The net effect of having high levels of cell surface expression of both PD-1 and PD-L1 on TILs and cancerous cells respectively is that the tumour is shielded from destruction by activated T cells. By blocking the interaction between PD-1 and PD-L1 with antibodies targeted against either protein, however, a powerful anti-tumour immune response is unleashed that can be harnessed for the treatment of cancer. Indeed, anti- PD-1/PD-L1 antibodies have been used in the clinic as an anti-tumour immunotherapy to treat a wide range of cancers including melanoma, non-small cell lung carcinoma, bladder cancer as well as relapsed Hodgkin's lymphoma [114-118].

Despite some incredible success stories, however, checkpoint blockade therapy has not been as effective as originally hoped. One reason for this lack of success is that PD-L1 is continuously trafficked between the cell surface and endosomes, which means that even in the presence of antibodies, it's not possible to disrupt all of the PD-1/PD-L1 interactions, preventing T cell activation. Therefore, considerable effort has been invested to find ways of reducing the overall levels of PD-L1 within cells as an alternative/complementary approach to targeting PD-L1 on the cell surface [119]. Recently, it has been reported that PD-L1 is palmitoylated on its cytoplasmic C-tail by zDHHC3 [120]. This covalent modification blocked PD-L1

ubiquitination suppressing its lysosomal degradation. The net effect of PD-L1 palmitoylation, therefore, is increased cellular levels of PD-L1. The authors subsequently showed that non-selective inhibition of PD-L1 palmitoylation with 2-bromopalmitate, knockdown of zDHHC3 as well as disruption of the zDHHC3/PD-L1 interaction with a peptide all had anti-tumour activity both *in vitro* and in mice bearing MC38 tumour cells. In summary, targeting PD-L1 palmitoylation may help to overcome the problem of PD-L1-mediated immune evasion in cancer.

Pharmaceutical targeting of protein palmitoylation for therapeutic purposes

Unlike kinases where numerous inhibitors have been created and tested for clinical efficacy in the treatment of disease [121], no therapeutically useful molecular modulators of protein palmitoylation have been developed thus far. This, in no small part, is due to a fundamental lack of knowledge regarding the molecular basis of both enzyme catalysis and substrate recruitment by DHHC-PATs. Going forward, even if isoform-specific active site inhibitors can be developed their usefulness in the clinic may be restricted due to the off-target effects they will cause as a consequence of blocking the palmitoylation of the entire substrate ensemble of the DHHC-PAT where they act and not just the protein of interest (Figure 1a). In contrast, if the recruitment of particular substrate proteins to specific DHHC-PATs could be selectively manipulated, it may be possible to obtain molecules that are therapeutically useful and had fewer side effects (Figure 1b). Similarly, although active site inhibitors of thioesterases such as APT1 and 2 have been developed and are incredibly useful pharmacological tools for lab applications [107-109, 122], they are unlikely to be used in people as the enzymes to which they bind depalmitoylate a wide range of proteins, and would be expected to cause numerous off-target effects. Whether or not the contact interfaces between particular thioesterases and specific substrates can be pharmaceutically targeted remains to be determined.

Efforts to identify small molecules that inhibit the activity of zDHHC-PATs have been on going for the last ten to fifteen years but thus far without much success [123-128]. In recent years, however, several novel screening assays have been devised that have allowed the identification of several small molecules that

block the palmitoylation of certain target proteins. For example, compounds that inhibit the auto-palmitoylation of *Erf2* (the *Saccharomyces cerevisiae* PAT responsible for catalysing the palmitoylation of Ras2, an ortholog of human Ras) activity were identified using a fluorescence-based coupled assay and validated utilising an orthogonal gel-based assay [129]. Furthermore, using a click chemistry approach, a high throughput screen run in 384-well format was used to identify inhibitors of human ras palmitoylation [130]. Most recently, an elegant screen based on fluorescence as a readout of subcellular localisation has been used to identify inhibitors of dual leucine-zipper kinase DLK palmitoylation, which are expected to be effective in limiting neurodegeneration following trauma [131]. Although these screens have identified molecules that alter the palmitoylation of particular target proteins, their mode of action (active site inhibitor versus substrate recruitment blocker) should be determined as part of assessing their potential clinical utility or otherwise.

Perspectives

Importance of the field

- The key role that palmitoylation plays in regulating a diverse range of cellular events potentially allows manipulation of numerous physiological processes by targeted intervention with either small molecules and/or peptides.

Summary of the current thinking

- Recent proof-of-principle studies have shown that the palmitoylation status of particular proteins can be selectively altered.

Future directions

- Existing drug-screening assays to identify molecular modulators of protein palmitoylation will most likely result in the discovery of active site inhibitors with broad spectrum activities which may restrict their clinical usefulness due to off-target effects.
- We favour targeting the recruitment of specific substrates to particular DHHC-PATs and/or APTs as we believe that this is the only way to

selectively alter the palmitoylation status of specific proteins, an absolute prerequisite for use in the clinic.

- To achieve this goal, a better understanding of how substrate proteins interact with both DHHC-PAT and APT enzymes is required, and assays will need to be developed that allow the identification of molecules that either promote or abrogate specific enzyme-substrate interactions.

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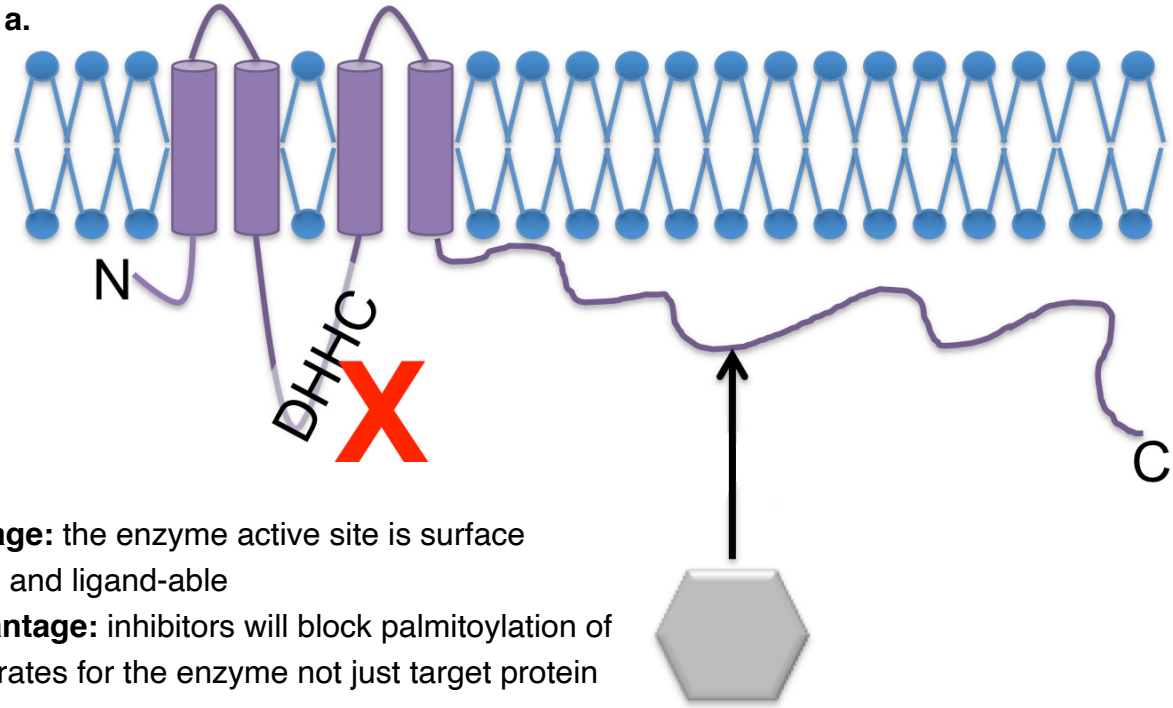
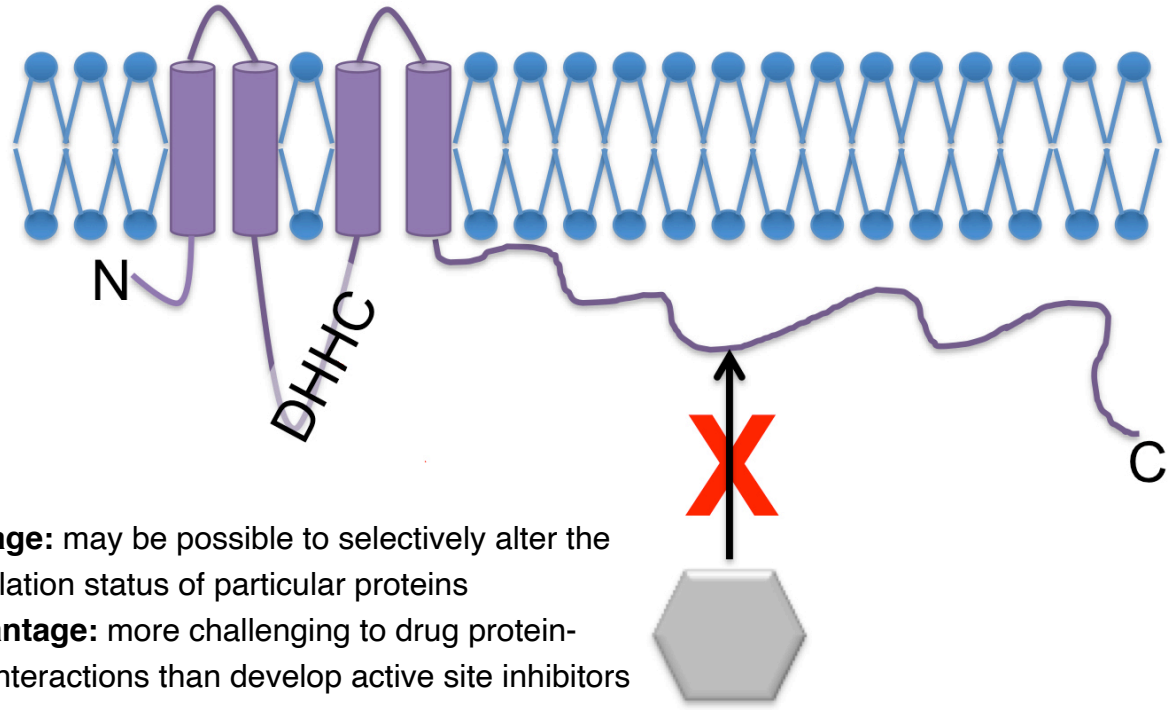
a.



Advantage: the enzyme active site is surface exposed and ligand-able
Disadvantage: inhibitors will block palmitoylation of all substrates for the enzyme not just target protein

b.



Advantage: may be possible to selectively alter the palmitoylation status of particular proteins
Disadvantage: more challenging to drug protein-protein interactions than develop active site inhibitors

Figure 1 Therapeutic targeting of zDHHC-PATs **a.** Targeting the active site, and **b.** Manipulating substrate recruitment